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Effects of laboratory maintenance on the nature of surface reactive antigens of *Neisseria gonorrhoeae*

R. J. ARKO, J. C. BULLARD, AND W. P. DUNCAN
From the Venereal Disease Research Branch, Center for Disease Control, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia, USA

Summary

The extensive *in vitro* cultivation methods used in propagating and maintaining gonococcal cells were found to affect their virulence, antigenicity, and ultrastructure. Adapting a laboratory-maintained strain of gonococci to animal virulence resulted in two lines of pilated cells with similar colonial morphologies. The animal-adapted cells, however, had a greater amount of extracellular pili and a more prominent peptidoglycan cell wall layer. They were also more resistant to the bactericidal effects of guinea-pig complement and more reactive in macroagglutination and bactericidal tests with strain-specific gonococcal antibody. In comparative guinea-pig protection trials, formalin-fixed cells of the animal-adapted cell line were 500 times more effective as immunogens than the laboratory-maintained cell line.

Introduction

The effectiveness of certain antigenic components of *Neisseria gonorrhoeae* in immunological procedures can be influenced by the media and methods used in gonococcal cell production and maintenance. A number of enriched laboratory media will readily support the growth of gonococci; however, the characterization of colony types (T) by Kellogg, Peacock, Deacon, Brown, and Pirkle (1963) and the later correlation of T1 and T2 with increased virulence for human volunteers (Kellogg, Cohen, Norins, Schroeter and Reising, 1968) has resulted in wide acceptance of a semisolid, translucent, enriched agar medium for making selective transfers of gonococcal colony types. Thayer and Martin (1966) added the antibiotics vancomycin, colistin, and nystatin (VCN) to a chocolate agar to produce a medium more selective for the isolation of pathogenic *Neisseria* species.

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Address for reprints: Dr R. J. Arko, Senior Veterinary Officr USPHS, Bldg 1, Room 3246, V.D. Research Branch, Center for Disease Control, Atlanta, Georgia 30333 USA

The effects of antibiotics and of extensive laboratory transfer on the antigenic composition and virulence of gonococcal isolates have been questioned for many years. Lack of a suitable infection model has, however, restricted the study of this problem.

In this research the recently developed mouse and guinea-pig models of gonococcal infection (Arko, 1972) were used to study the virulence, antigenic reactivity, and ultrastructure of a gonococcal isolate which has been maintained by selective serial transfers on laboratory media for over 5 years.

Material and methods

In our laboratory the N9 isolate of gonococci has been used in numerous immunological studies involving humans, chimpanzees, and laboratory animals. The growth and maintenance of this isolate require the selective transfer of colonies on to a Gc Base (GcB) medium, enriched with a defined supplement, Isovitalex (Baltimore Biological Laboratories, Baltimore, Md. USA) with or without selective VCN antibiotics. The harvested colonies are preserved by either lyophilization or freezing at — 70°C. This isolate has been transferred at least 300 times since its isolation from an infected patient.

Using the T1 and T2 colonies of the laboratory-maintained N9 isolate, we obtained subcutaneous chamber infections in mice which were immuno-suppressed with 0·5 mg. dexamethazone (Schering Co., Bloomfield, N.J., USA) 1 day before and daily for 3 days after gonococcal inoculation (Arko, 1973). The N9 isolate was prepared for animal inoculation by streaking the desired colony types on to agar GcB medium and incubating the plates for 20 hrs at 36°C in a candle-extinction jar. The surface growth on these plates was harvested with a sterile cotton swab and suspended in trypticase soy broth (TSB 30 g/litre of distilled water), and the suspension was adjusted to an optical density (OD) of 0·5 in a 13 × 100 mm glass tube with a Leitz Spectrophotometer set at 535 nm. The suspension of cells was then injected into mouse subcutaneous chambers in 0·2-ml amounts. The inoculum dose was quantitated by making 10-fold serial dilutions of the stock suspension and inoculating three plates of GcB medium with 0·1-ml amounts from each of the 1×10^{-5}, 1×10^{-6}, and 1×10^{-9} dilutions. The colony forming units (CFU) of gonococci were counted on each plate after incubation for 24 hrs at 36°C in a candle-extinction jar. Fluid specimens of 0·05 ml were obtained from each

mouse chamber at 1, 3, 6, 8, and 24 hrs after injection and streaked on to plates of GcB medium. Growth from these plates was re-suspended in TSB and injected into subcutaneous chambers in additional mice. After chamber adaptation of the T1 cells of N9, a chamber-infective dose 50 per cent. (CID_{50}) was determined by injecting known numbers of gonococci into the chambers of non-immunosuppressed (normal) mice. Fluid was withdrawn from each mouse at 3-day intervals and streaked on to GcB plates which were incubated as previously described. The CID_{50} was determined by graphic interpolation between the CFUs of gonococci producing more than and less than 50 per cent. infection rates.

The survival *in vivo* and *in vitro* of the laboratory-maintained and mouse-adapted T1 cells of N9 were compared in normal mouse chambers and in phosphate buffered saline (PBS), pH 7·2. Cells of T1 colonies from both the laboratory-maintained and mouse-adapted N9 lines were suspended in PBS to optical density 0·5 levels, and 0·2-ml amounts of each were immediately injected into two groups of four mice each. The remaining suspensions of cells were maintained in PBS at 35°C for up to 24 hrs. Specimens were obtained from each mouse chamber and from the PBS suspension at 1-, 3-, 6-, 8-, and 24-hr intervals after injection and streaked on GcB plates.

A guinea-pig immunization study was conducted with three formalin-fixed immunogens prepared from the T1 colonies of the laboratory-maintained and mouse-adapted N9 cell lines. Thirty 100 × 15 mm agar plates of GcB medium + Isovitalex were heavily streaked with the mouse-adapted N9 T1 cells.After incubation at 36°C for 20 hrs in a candle-extinction jar, these plates were removed and examined microscopically to determine the colony types present. All plates contained more than 95 per cent. T1 colonies and were immediately harvested with individual glass 'L' rods into three weighed centrifuge tubes, each containing 50 ml sterile 0·85 per cent. saline. The three tubes were centrifuged at 1,500 G for 30 min. The supernate was removed and the tubes were reweighed. The wet weight of cells in each tube was determined in grams and adjusted to 5 per cent. (wet cell weight/volume) with the supernate which was previously removed from each tube. A 2-ml aliquot of the 5 per cent. cell suspension was removed from each tube and diluted 1: 5 with sterile saline containing 5 per cent. neutral formalin. This portion of cells was kept at 4°C and used in a macroscopic agglutination test for gonococcal antibodies (Arko, 1974). The remaining cell suspension was fixed with 0·1 per cent. neutral formalin and held at 4°C until injected into guinea-pigs.

A series of 41, 250-g., male, Hartley strain guinea-pigs was randomly divided into four groups, three of which received immunizations with the above individual immunogen preparations. Each animal was given four 1-ml intramuscular injections of the respective immunogen at 1 week intervals. The fourth group served as non-immunized challenge controls. Three days after the fourth injection, two stainless steel chamber implants were placed in each guinea-pig of each group (Arko, 1973). Seven days later the resistance of each guinea-pig was tested by inoculating a known number of guinea-pig-virulent N9 T1 cells into the subcutaneous chambers. The guinea-pig chambers were cultured at 3-day intervals after challenge and culture-negative chambers were rechallenged the following day with a 10 to 100-fold increase in inoculum CFUs.

The antigen sensitivity and specificity of the laboratory-maintained and mouse-adapted N9 T1 cells for gonococcal antibodies to the N9 isolate were compared by a previously described microbactericidal test of Tramont, Sadoff, and Artenstein (1974) and by a macroagglutination procedure. The A, G, C, and N9 immune sera in the tests were obtained from guinea-pigs previously infected in subcutaneous chambers for 15 days or longer with the respective gonococcal isolate.

The T1 cell ultrastructure of the laboratory-maintained and mouse-adapted N9 cell lines was studied by electron microscopy to determine if any morphological differences could be found to correlate with the virulence variation observed in the mouse chambers. T1 cells of the laboratory-maintained and mouse-adapted cultures, grown on plates of GcB medium for 18 hrs at 36°C or in a liquid medium supplemented with 2 per cent. yeast extract (Frantz, 1942), and cells in fluid from infected mouse chambers were examined by whole-mount and ultrathin-section preparation. Whole mounts were prepared by picking T1 colonies from GcB plates with a sterile platinum loop and suspending the cells in sterile distilled water. A drop of the suspension was placed on Formvar-coated copper grids, blotted dry, negatively stained with 2 per cent. aqueous uranyl acetate, pH 4.0, for 15 sec., again blotted dry, and examined with a Philips model 200 transmission electron microscope operating at an accelerating voltage of 60 kV.

For thin sections the organisms were collected with sterile cotton swabs or by centrifugation (1,500 G for 10 min.) and suspended in 2 per cent. glutaraldehyde in 0·1 Mol sodium cacodylate buffer, pH 7·4, for 2 hrs at room temperature. A pellet was formed by centrifuging in a Servall Super Speed centrifuge at 16,000 G for 15 min. The pellets were suspended in a 0·1 Mol sucrose cacodylate buffer rinse at 4°C overnight. They were post-fixed in 1 per cent. buffered osmium tetroxide for 1 hr at room temperature, centrifuged as before, dehydrated in graded ethanols, infiltrated with propylene oxide, and embedded in Maraglas.

Ultrathin sections were cut on a Servall Porter-Blum MT-1 ultra-microtome, picked up on bare copper grids, stained with uranyl acetate and lead citrate (Reynolds, 1963), and examined in the electron microscope.

Results

The laboratory-maintained T1 cells of N9 did not survive more than 6 hrs when 1×10^7 CFU or less was injected into the subcutaneous chambers of normal mice. However, mouse-chamber infection was obtained in dexamethasone-treated mice by injection and subpassage of cells on GcB plates at 6-hr intervals. Once an infection had persisted 5 days or longer the N9 T1 cells became highly virulent for normal mouse subcutaneous chambers. A mouse CID_{50} of approximately 1×10^2 CFU was determined for the T1 cells after mouse adaptation.

When we compared the laboratory-maintained and mouse-adapted cultures of N9 T1, we found that the

in vitro survival time for both was approximately 8 hrs for 1×10^7 cells per ml in 35°C PBS. In contrast, the mouse-chamber survival time for the laboratory-maintained N9 was 6 hrs or less, but the mouse-adapted N9 cells remained viable for up to 30 days.

In preparing formalinized immunogens from the N9 T1 cells, differences in cell mass yield were noted between the laboratory-maintained and mouse-adapted cells grown on GcB medium and between the mouse-adapted cells grown on GcB and GcB + VCN media. The laboratory-maintained N9 cells grown on thirty plates of GcB medium yielded only 2·7 g T1 cells, in comparison to the mouse-adapted cells

TABLE I *Relative resistance of normal and N9 immunized guinea-pigs to chamber infection with virulent N9 type 1 cells of* N. gonorrhoeae

Formalin-fixed immunogen[a]	*Immunogen culture medium*	*Challenge in CFU*[b] *(Number infected/number challenged)*[c]					*Interpolated guinea-pig ID*$_{50}$ *(in CFU)*
		$2{\cdot}0 \times 10^2$	$1{\cdot}0 \times 10^4$	$2{\cdot}5 \times 10^5$	$4{\cdot}0 \times 10^6$	$5{\cdot}0 \times 10^7$	
Mouse virulent N9 T1 cells	Gc base + Isovitalex	0/11	2/11	2/9	4/7	0/3	500,000
Mouse virulent N9 T1 cells	Gc base + VCN[d] + Isovitalex	0/12	7/12	5/5	NT	NT	10,000
Laboratory mouse avirulent N9 T1 cells	Gc base + Isovitalex	0/8	7/8	1/1	NT	NT	1,100
Normal controls	None	10/10	NT	NT	NT	NT	20

[a]Immunization consisted of giving four 1-ml intramuscular injections of each immunogen at 1 week intervals
Each immunogen was prepared to contain 5 per cent. (wet cell wt/volume) of pilated type 1 cells fixed with 0·1 per cent. formalin.
[b]Challenge was initiated 10 days after the last immunization by injecting a 0·2 ml suspension containing the indicated number of colony forming units (CFU) of gonococci into the right subcutaneous chamber of each animal.
[c]Chamber fluid specimens were removed at 3-day intervals and cultured on Gc base medium; culture negative animals were rechallenged with the next larger number of gonococci on the following day.
[d]VCN antibiotic inhibitors and Isovitalex were obtained from BBL, Cockeysville, Maryland, USA

TABLE II *Reactivity of mouse avirulent laboratory-maintained and mouse-adapted type 1 cells of N9 gonococci in agglutination and bactericidal serological procedures*

Titre	*Antigens used*	*Normal serum*	*Guinea-pig Gc antisera*[b]			
			A	*G*	*C*	*N9*
Macroagglutination[a]	Laboratory-maintained N9 cells	—	—	—	—	1:4
	Mouse-virulent N9 cells	—	1:4	—	—	1:64
Serum bactericidal[c]	Laboratory-maintained N9 cells	1:8	1:16	1:8	1:16	1:8
	Mouse-virulent N9 cells	—	1:8	1:8	1:16	1:256

[a]Macroagglutination of formalin-fixed type 1 cells read after 60 min. incubation with antisera
[b]Gonococcal antisera was obtained from guinea-pigs having recovered from chamber infections with the respective serotype of gonococci
[c]Serum microbactericidal titre was determined as the dilution of serum which produced a 50 per cent. reduction in cell survival after incubation for 90 min. with test antigen

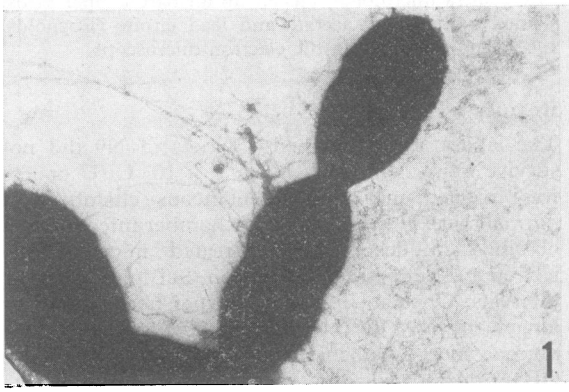


FIG. 1 *Negative stain of whole mount preparation of the mouse-adapted N9 cells, showing 'bullet-shaped' pilated diplococci.* × 18,750

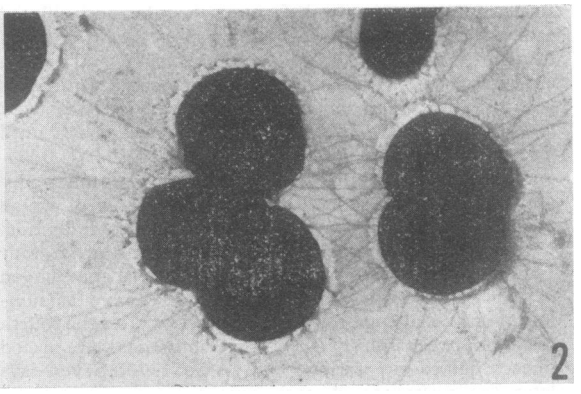
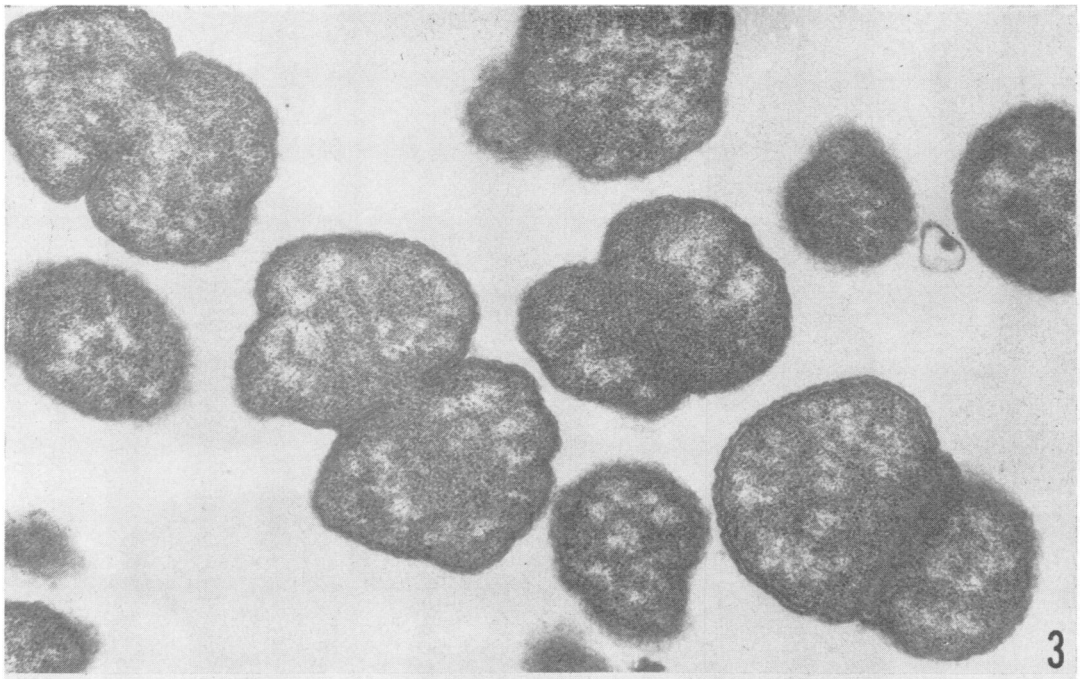


FIG. 2 *Negative stain of laboratory-maintained N9 cells, showing pilated diplococci.* × 18,750



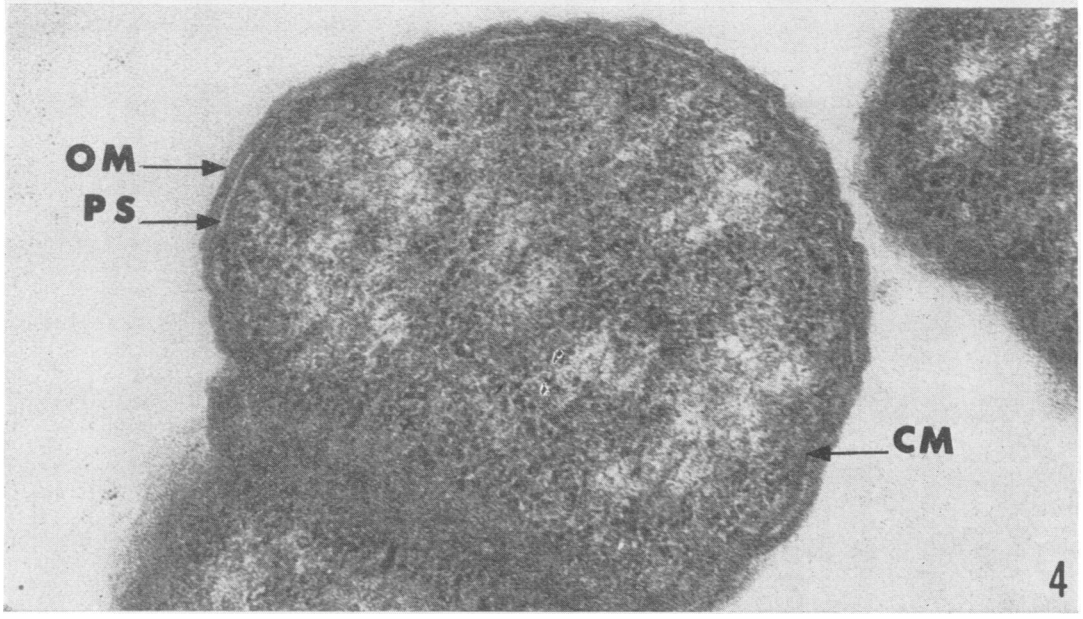


FIG. 3 *Ultrathin section of laboratory-maintained N9 T1 cells* × 41,125

FIG. 4 *Higher magnification of ultrathin section of laboratory-maintained N9 T1 cells.*
OM = outer cell membrane, PS = periplasmic space, CM = cytoplasmic membrane. × 88,125

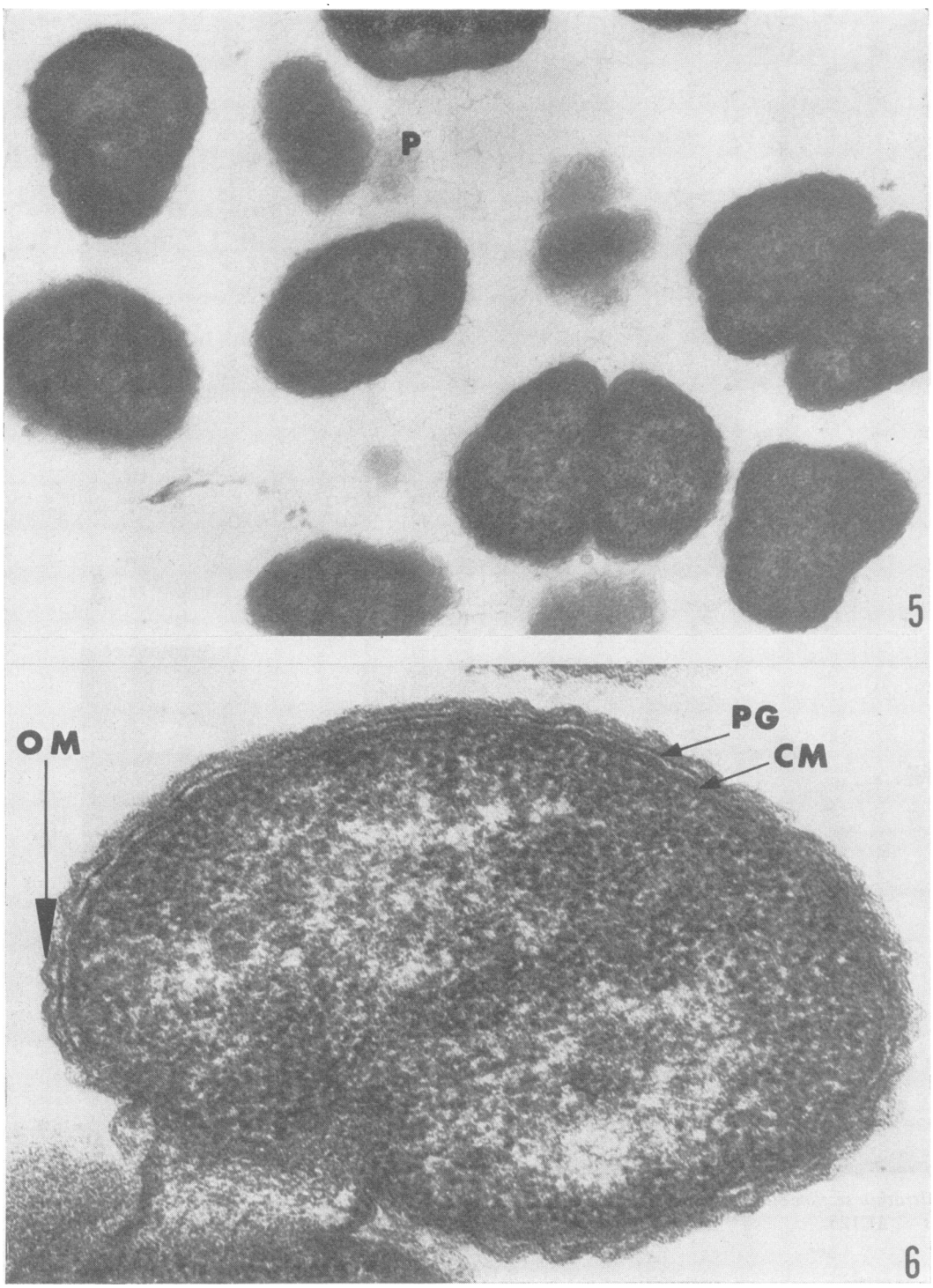


FIG. 5 *Thin section of N9 T1 mouse-adapted cells. P = aggregates of pili.* × 41,125

FIG. 6 *Thin section of mouse-adapted N9 T1 cells. OM = outer cell membrane, PG = peptidoglycan layer, CM = cytoplasmic membrane.* × 141,000

which yielded 5 g. However, the mouse-adapted cells produced only 2·6 g when VCN was incorporated into the growth medium.

Guinea-pigs receiving 50 mg of the three above immunogens developed tissue swelling of approximately 3 by 1 cm at the injection site within 48 hrs. The tissue response persisted in most animals for 4 to 7 days. The postimmunization challenge results for the three immunogen preparations tested in guinea-pigs are shown in Table I. The results of serological tests with antigens prepared from N9 T1 cells of the laboratory and mouse-adapted cultures are shown in Table II.

In the electron microscopic study we compared characteristics in the appearance of T1 cells from both the laboratory-maintained and animal-virulent N9 cultures. When incubated for 18hrs on a semisolid GcB medium and negatively stained, T1 cells from these cultures displayed abundant surface pili (Figs 1 and 2), as previously described by Jephcott, Reyn, and Birch-Anderson (1971) and Swanson, Kraus, and Gotschlich (1971). However, the animal-virulent T1 cells sectioned directly from the chamber fluid of mice that had been infected for 72 hrs or longer were devoid of attached surface pili. As shown in Fig. 2, T1 cells of the laboratory-maintained N9 culture grown on GcB and negatively stained appeared as typical diplococci, but T1 cells from the animal-virulent culture prepared under the same conditions appeared pleomorphic or 'bullet-shaped' (Fig. 1).

In ultrathin sections of the laboratory-maintained N9 cells grown on GcB medium for 18 hrs, the cell wall appeared to consist of a relatively thick, double-lined outer membrane separated by an irregular periplasmic space from a double-lined cytoplasmic membrane (Figs 3 and 4). Relatively few extracellular pili were seen in thin sections of the laboratory-maintained N9 culture. Thin sections prepared under the same conditions from the animal-virulent cultures of N9 showed much larger amounts of extracellular pili as well as greater electron density of the cell wall layers (Figs 5 and 6). In addition, the peptidoglycan layer, a dense granular lamina between the outer and cytoplasmic membranes (Fig. 6), showed much greater development than in the laboratory-maintained culture (Fig. 4). Although pili appeared on T1 cells grown on semisolid medium, they were not present on cells in sections of gonococci grown in Frantz liquid medium (Fig. 7) or in infected animal fluid (Fig. 8).

Thin sections prepared from the animal-virulent

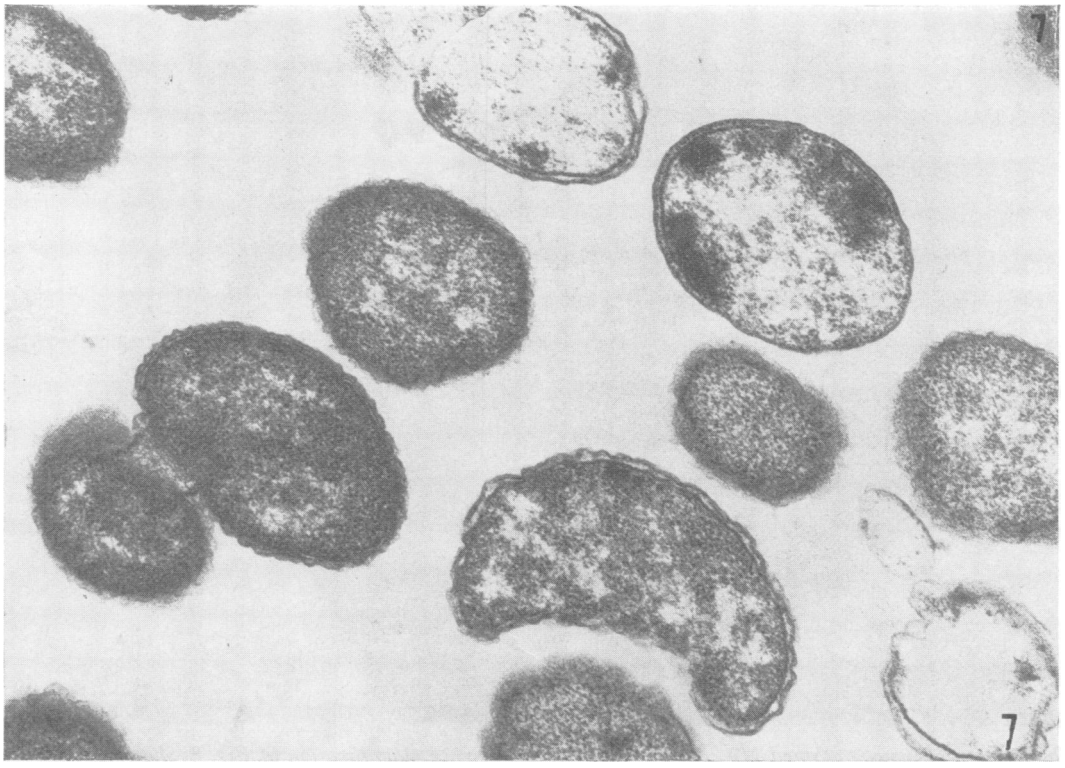
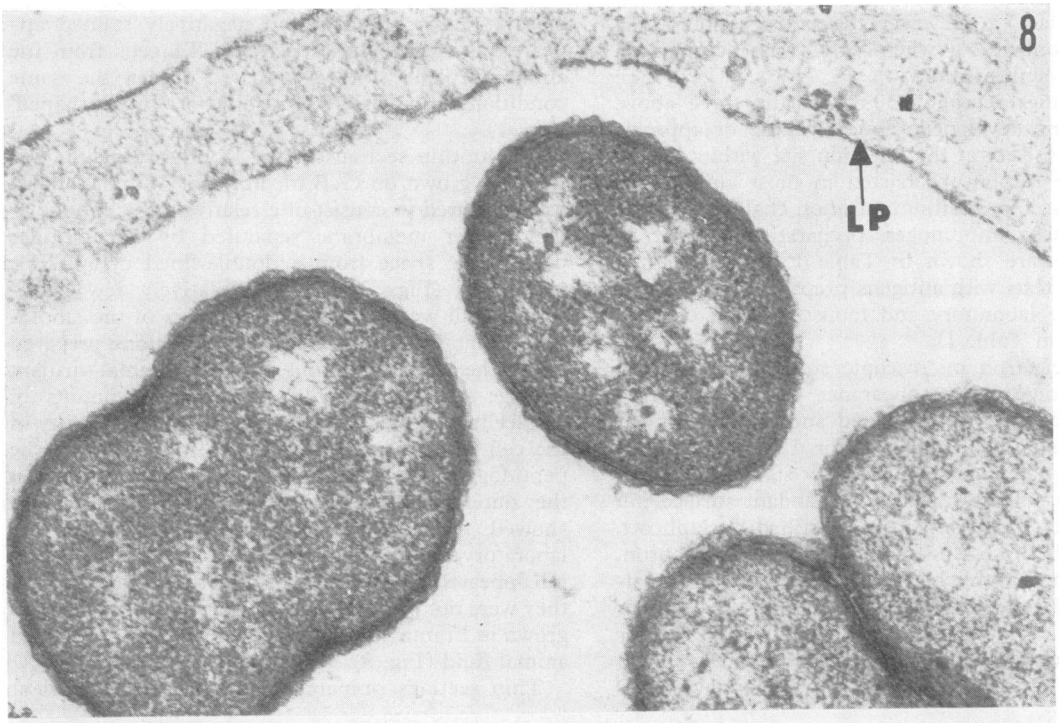


FIG. 7 *Thin section of mouse-adapted N9 T1 cells grown in Frantz medium, showing marked cellular pleomorphism and autolysis.* × 41,125



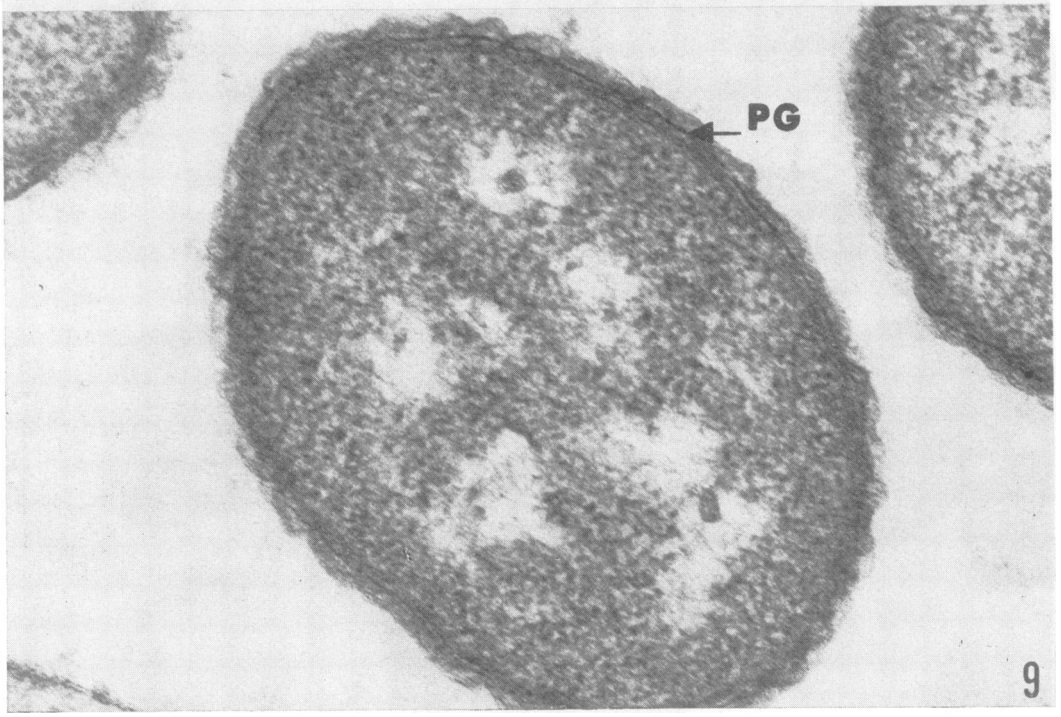


FIG. 8 *Thin section of mouse-adapted N9 cells in infected mouse chamber fluid plus a portion of a leucocyte pseudopod LP.* × 64,050

FIG. 9 *Higher magnification of Fig.* 8, *showing prominent peptidoglycan layer PG.* × 128,100

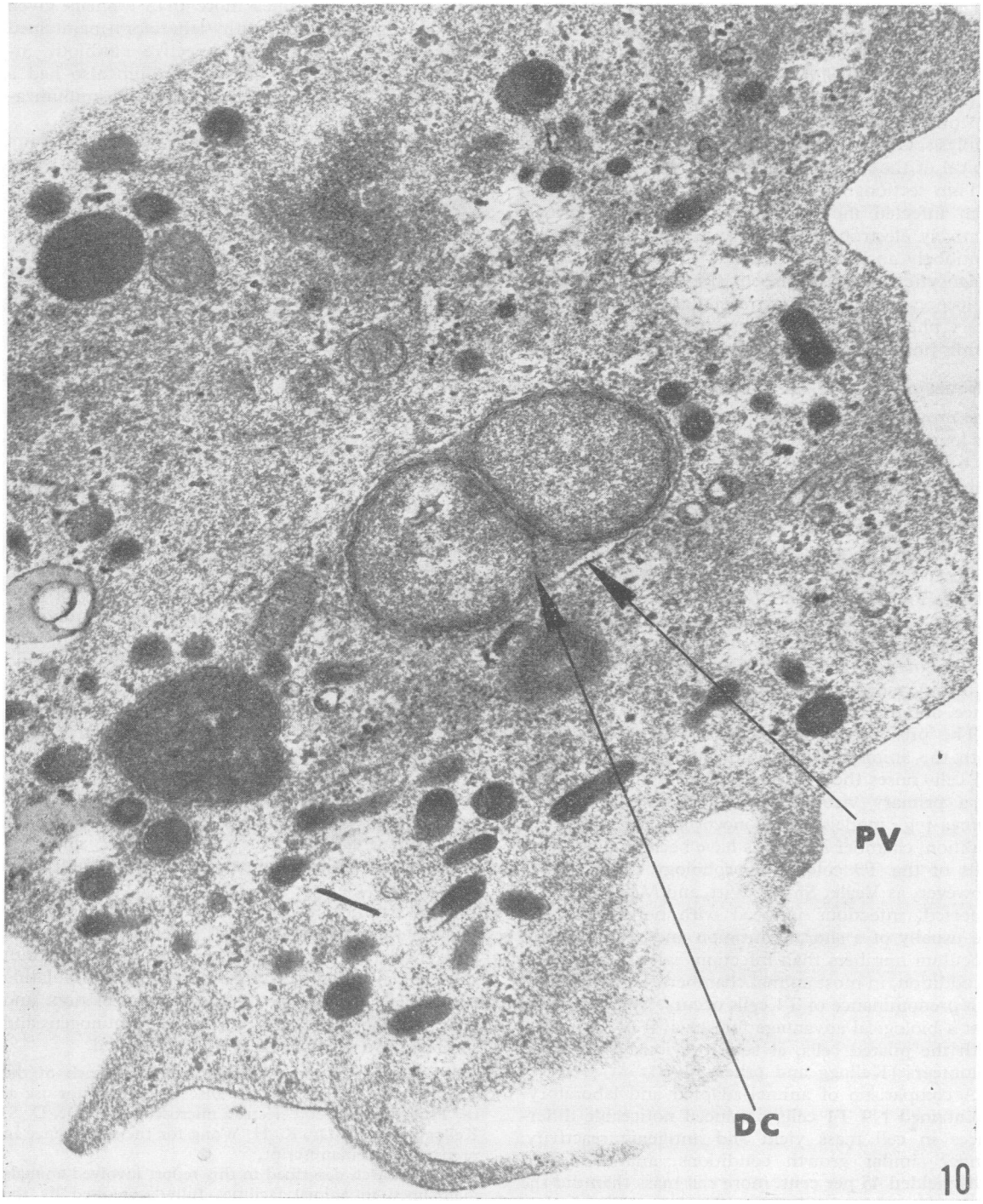


FIG. 10 *Thin section of a leucocyte in mouse chamber fluid containing engulfed diplococci DC. PV = phagocytic vacuole.* × 37133

N9 cells grown for 18 hrs in the Frantz (1942) liquid medium showed greater variation in cellular morphology and structure (Fig. 7) than did similar cells grown on a semisolid GcB medium (Fig. 5). Many of the atypical cells (Fig. 7) do not appear to have a peptidoglycan layer and seem to be undergoing autolysis. Other cells appear to have cell wall structure typical of the animal-virulent T1 cells.

Thin sections made of diplococci present in fluid from infected mouse chambers appear to have a relatively electron-dense cell wall with a prominent peptidoglycan layer (Figs 8 and 9). Although mouse phagocytic cells were present (Figs 8 and 10), most diplococci were found extracellularly. However, an intact phagocyte with at least two gonococcal cells within phagocytic vacuoles may be seen in Fig. 10.

Discussion

The propagation and maintenance of gonococcal cells for long periods by the selective transfer of colonies on a conventional GcB medium may, as shown in Tables I and II, have a demonstrable effect on their virulence and antigenicity. The laboratory-maintained N9 T1 cells in this study retained their typical colonial morphology; however, their continuous propagation *in vitro* may have contributed to their relative loss of virulence for chambers in laboratory animals. Adaptation of the laboratory-maintained N9 culture to virulence for animal chambers has resulted in two lines of T1 cells which are indistinguishable in colony morphology but have differences in virulence, antigenicity, and cell ultrastructure.

The presence of surface pili (Figs 1 and 2) on both the animal-adapted and laboratory-maintained T1 cells raises the question of the importance of pili as a primary virulence factor in the mouse and guinea-pig models of gonococcal infection. In addition, chamber infections have been obtained with cells of the T3 colonial morphology (Arko, 1974). However, as Veale, Smith, Witt, and Marshall (1975) reported, infections induced with nonpilated cells are usually of a shorter duration and require larger inoculum numbers than infections with pilated cells. In addition, in most animal chambers, a gradual shift to a predominance of T1 cells occurs, which suggests that a biological advantage for survival *in vivo* exists with the pilated cells, as seen previously in human volunteers (Kellogg and others, 1963).

A comparison of animal-adapted and laboratory-maintained N9 T1 cells produced noticeable differences in cell mass yield and antigenic reactivity. Under similar growth conditions, animal-adapted cells yielded 45 per cent. more cell mass than did the laboratory-maintained T1 cells. In addition, they were more effective in stimulating protective immunity. Guinea-pigs immunized with virulent T1 cells (Table I) resisted challenge with 25,000 times more gonococci than nonimmunized animals, and approximately 500 times more than animals given similar immunization with laboratory-maintained T1 cells. The addition of selective antibiotic inhibitors, VCN, to the culture medium also had a detrimental effect on cell mass yield and immunization effectiveness (Table I).

Gonococcal T1 cells produced by culture for 18 hrs in Frantz liquid medium supplemented with 2 per cent. yeast extract have also been found to be less effective immunogens when compared with similar cells harvested from a semisolid GcB medium (Arko, unpublished data). As shown in Fig. 7, approxiproximately 50 per cent. of the cells harvested from the Frantz medium have an atypical cell wall structure and appear to be autolysing, which may have contributed to their decreased effectiveness as immunogens.

In thin sections, the degree of development of peptidoglycan layer appeared to be the most differentiating cell characteristic between animal-adapted and laboratory-maintained cells. The prominent peptidoglycan layer of the animal-adapted N9 cells (Figs 6 and 9) is similar to that described by Novotny, Short and Walker (1975) for gonococcal cells found in human pus and for cell walls of *Acinetobacter* (Thornley, 1975). Although the peptidoglycan layer is believed to be weakly antigenic and to function primarily in cell morphology, it has not as yet been isolated or tested as a gonococcal immunogen. However, Buchanan and Arko (in preparation) found that the closely associated cell outer membranes and antigens isolated from them are capable of stimulating strain-specific, bactericidal antibodies in guinea-pigs, which were subsequently demonstrated to have a significant level of strain-specific immunity to the immunizing isolate of gonococci. In addition, the level of serum bactericidal and indirect fluorescent antibody has recently been demonstrated to have a positive correlation with the relative resistance of immunized male chimpanzees to urethral and pharyngeal gonococcal infection (Arko, Duncan, Brown, Peacock, and Tomizawa, 1976). It appears that, in these animal models, other surface reactive antigens, in addition to pili, may play an important role in gonococcal cell virulence and in stimulating protective antibodies. The further isolation and characterization of these antigens as immunogens and serological test reagents seems warranted.

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The research described in this report involved animals maintained in animal facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

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